

## Binding Features of Ethidium Bromide and Their Effects on Nuclease Susceptibility of Calf Thymus DNA in Presence of Spermine

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Functional properties of enzyme or some other agents acting on nucleic acids may often be dependent upon the conformation of the nucleic acids.<sup>1,2</sup> Previously several papers reported that in absence of any cell growth, the nucleotide degradation of yeast mit-DNA into vegetative "petits" was induced when the yeast mit-DNA was acted on by ethidium bromide (EtBr).<sup>3-6</sup> On the other hand, we could observe a change in binding cooperativity of ethidium with calf thymus DNA in the presence of spermine at the concentration where the DNA collapses into condensed state.<sup>7</sup>

These data prompted us to undertake present work testing the effect of ethidium bromide on the susceptibility of calf thymus DNA for DNase 1 in the absence and presence of spermine at the concentration where the DNA collapses, with the intent of developing probes to study the structural transition of DNA in its monomolecular condensation process induced by spermine. The present study demonstrates spermine effect on ethidium-dependent DNA susceptibility to DNase 1 correlated with altered features of ethidium binding to DNA.

### Experimental Section

**Materials.** Calf thymus DNA (Type 1), RNA (purified from Baker's yeast, Type XI), deoxyribonuclease 1 (DNase 1, DN-100), ribonuclease A (RNase A, Type 1-AS), and spermine tetrahydrochloride were purchased from Sigma Chemical Company and used without further purification. Ethidium bromide (Sigma) was recrystallized once with methanol prior to use. The heat denatured DNA was prepared by heating the DNA ( $OD_{260} = 1.4$ ) dissolved in 8 mM citrate buffer, pH 7, in a boiling water bath for 20 min, and then cooling rapidly to 0 °C in an ice-water. The DNA concentrations are expressed in terms of nucleotide phosphate by using the extinction coefficient of  $\epsilon_{260} = 6,600 \text{ M}^{-1}\text{cm}^{-1}$ . The concentration of ethidium bromide (EtBr) was determined spectrophotometrically by using the extinction coefficient  $\epsilon_{476} = 5,680 \text{ M}^{-1}\text{cm}^{-1}$ .

**Test of the susceptibility of DNA to nucleases.** In the DNase 1 reaction, the procedure of Kunitz with some modifications was employed.<sup>8</sup> The final concentration of the ace-

tate buffer components in the reaction mixture of 2 mL was: 30 mM  $\text{MgCl}_2$ , 5 mM NaCl, and 8 mM acetate, and appropriate concentration of spermine either included or omitted. Susceptibility of DNA to DNase 1 was expressed as the increase of absorbance at 260 nm of the supernatant of the reaction mixture containing digestive DNA after discarding pellet by centrifugation. Further experimental details are described in the reference.<sup>7</sup> RNA degradation by RNase A was measured by the methods of McDonald<sup>9</sup> with some modifications. The enzyme reaction was initiated by adding and mixing 0.1 mL RNase A solution preincubated at 20 °C with 1.9 mL of substrate solution in the acetate buffer described above.

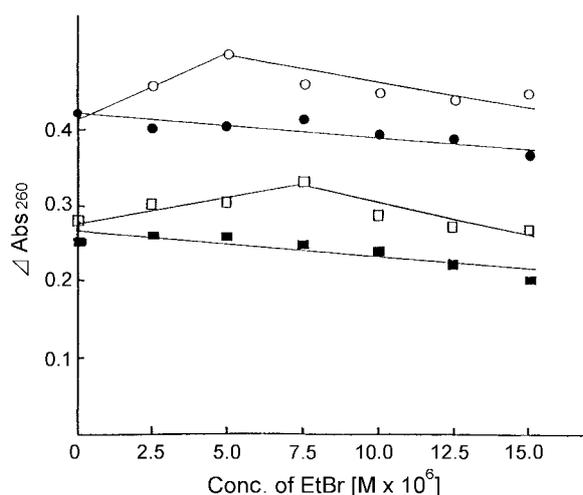
### Spectroscopic titration of ethidium binding with DNA.

Total constant concentration of ethidium bromide ( $7.0 \times 10^{-6} \text{ M}$ ) was titrated at the wavelength of 520 nm with varying concentration of DNA in the phosphate buffer, pH 7.0, in either presence ( $3.3 \times 10^{-4} \text{ M}$ ) or absence of spermine at 20 °C using Pye Unicam SP 1,800 spectrophotometer. Hypochromicity and red shift to 520 nm with isobestic point at 510 nm were observed in the ethidium bromide binding to DNA. Free ethidium bromide has maximum absorption at 476 nm.

### Results and Discussion

In our previous work, we suggested the possibility that the overall tertiary structure rather than secondary structure of DNA is affected more sensitively by the intercalation of ethidium bromide between the base pairs of DNA.<sup>10-12</sup> This means that causal limited introduction of ethidium bromide may induce subtle overall three-dimensional structural transitions of the DNA in its monomolecular condensed state complexed with spermine.<sup>10</sup> According to the previous reports, DNA duplex lengthening and unwinding is caused by the intercalative binding of ligands.<sup>13</sup> In addition, a kink in the DNA structure is formed as the result of the intercalation.<sup>13</sup> Because of the kink, a DNA fully saturated with ethidium will be forced into a superhelical structure.<sup>14</sup> Since DNA collapsed into a condensed state is confined to a volume smaller than in free solution (in absence of spermine), such an increase in length might be unfavorable. Here we are interested in finding how such a structural transition in DNA could be probed by its susceptibility to the DNase 1.

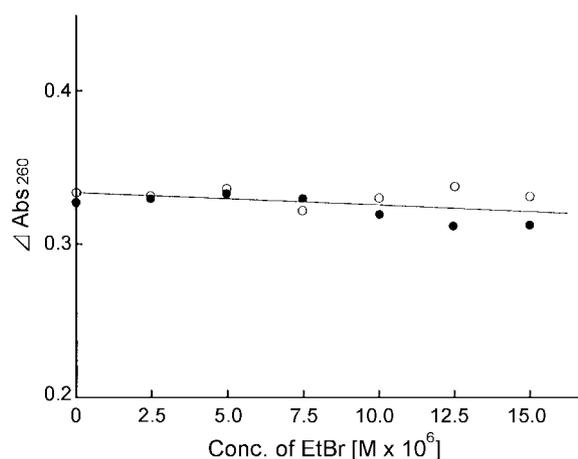
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**Figure 1.** Ethidium-dependent differential susceptibility of the native DNA and the denatured DNA in presence ( $3.3 \times 10^{-4}$  M) and absence of spermine. The DNA concentration is  $1.2 \times 10^{-4}$  M. Further experimental details are as described in the text.  $\circ$ , native DNA minus spermine;  $\bullet$ , denatured DNA minus spermine;  $\square$ , native DNA plus spermine;  $\blacksquare$ , denatured DNA plus spermine.

In Figure 1, which shows the ethidium-dependent DNA susceptibility to DNase 1 in presence and absence of spermine, we can see that there is an optimum ethidium bromide concentration in both free solution and in condensed state (in presence of spermine). (In our subsequent statement of 'in the presence of spermine', the spermine concentration of  $3.3 \times 10^{-4}$  M, where the DNA is collapsed into a condensed state, is meant otherwise mentioned). Without spermine, the rate of increase of the DNA susceptibility, as the ethidium bromide concentration is increased, is larger and reaches the optimum ethidium bromide concentration earlier than that in the presence of spermine. In both cases of the presence and absence of spermine, the profile of the DNA susceptibility to the DNase 1 vs. ethidium concentration is biphasic. In the range of low level of ethidium bromide, the susceptibility is enhanced with the increase of the concentration of ethidium bromide, whereas the susceptibility is lowered as the ethidium bromide concentration is increased beyond the optimum concentration. In contrast with the native DNA, there is no optimum concentration of ethidium bromide in case of denatured DNA, and the susceptibility of the denatured DNA is slowly decreased as the ethidium bromide concentration is increased. As shown in Figure 2, susceptibility of RNA to the RNase A in the presence and absence of spermine was monophasic and seems to be independent of ethidium bromide. From these observations, we may infer that there is no stacking or partial stacking of ethidium to RNA due to its lack of DNA-like double helix structure, and it shows similar susceptibility pattern to that of the denatured DNA in Figure 1.

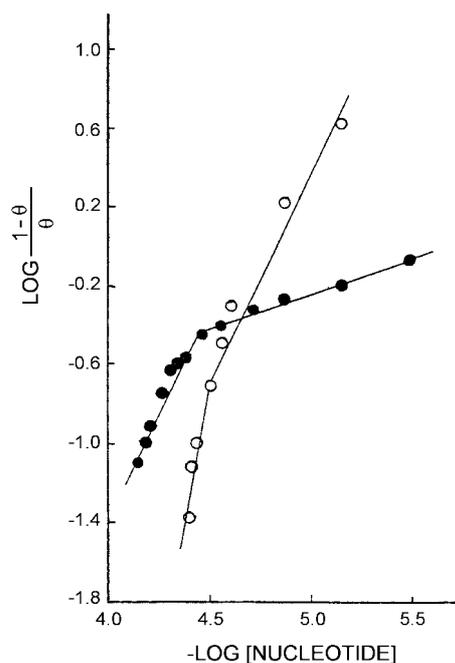
Two major binding modes of ethidium bromide to DNA have been well described.<sup>13</sup> A strong binding mode, the 'intercalative binding', is caused by the intercalation of ethidium bromide between Watson-Crick base pairs of



**Figure 2.** Ethidium-dependent RNA susceptibility for RNase A in presence ( $\bullet$ ) and absence ( $\circ$ ) of spermine.

DNA. It is also shown to be anticooperative, binding preferably at smaller ethidium bromide to DNA concentration ratio. As the second binding mode, a weak binding which is the 'nonintercalative binding' occurs only after the saturation of the intercalative sites and shows a positively cooperative binding.<sup>13</sup>

Here, we looked into the modes of ethidium-binding with the DNA in both presence and absence of spermine to see how the binding modes are related to the effects of ethidium bromide on the DNA susceptibility to DNase 1. Empirical Hill plots of ethidium bromide binding with DNA in the presence and absence of spermine are shown in Figure 3. The plots of both are discontinuous at the ethidium bromide concentration which corresponds to the ratio of D/P of about 0.2 (D/P: the ratio of the concentration of ethidium bromide



**Figure 3.** Empirical Hill plot of ethidium bromide (EtBr) binding to the DNA in presence ( $\bullet$ ) and absence ( $\circ$ ) of spermine.

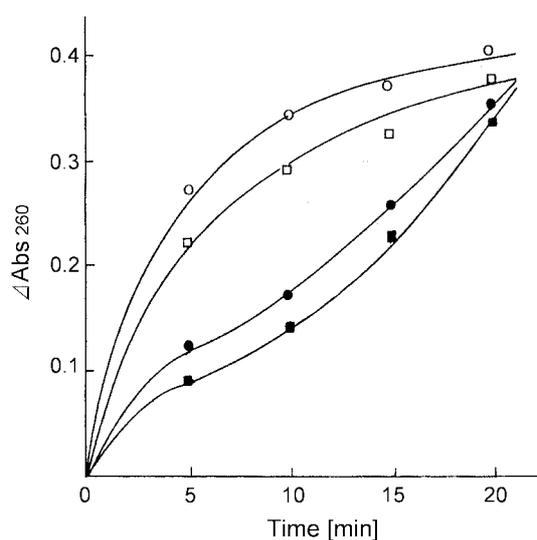
**Table 1.** Hill coefficient (n) of ethidium bromide binding to calf-thymus DNA in presence ( $3 \times 10^{-4}$  M) and absence of spermine\*

	n (minus spermine)	n (plus spermine)
Low EtBr Conc.	0.2	0.5
High EtBr Conc.	0.4	3.0

\*By titrating with varying concentration of DNA in presence and absence of spermine, the increase of fractional absorbance ( $\theta$ ) at 520 nm were obtained. If the normalized increase in absorbance during the transition can be equated with the quantity, the empirical Hill plot can be derived as follows. Hill Plot.  $\log(\theta/(1-\theta)) + pK_{app} = -1/n \log[\text{nucleotide}]$ . where, n: Hill coefficient,  $\theta$ : Fractional occupancy of the EtBr binding sites,  $K_{app}$ : Apparent dissociation constant

to that of DNA nucleotides). At the higher ethidium bromide concentrations beyond the breaking point, the Hill coefficients are increased in both cases, being the increase rate in presence of spermine much greater than that in absence of spermine. As shown in Table 1, the Hill coefficient changes from 0.2 to 0.4, whereas the value changes from 0.5 to 3 with spermine. We notice that these results correlate well with the data of ethidium-dependence of the susceptibility of DNA to DNase 1 in the presence and absence of spermine shown in Figure 1, and are related with the two binding modes of ethidium bromide to DNA referred above.

Finally, as a probe to test the possibility of altered mode of nucleolytic susceptibility of DNA, dependent upon its conformational changes induced by the binding of spermine, the spermine-dependent time course of the liberation of acid-

**Figure 4.** Spermine-dependent time course of liberation of acid-soluble products from the DNA by DNase 1 in presence ( $1.5 \times 10^{-5}$  M) and absence of ethidium bromide (EtBr). ○, minus spermine without EtBr; ●, plus spermine without EtBr; □, minus spermine with EtBr; ■, plus spermine with EtBr.

soluble products from the DNA by DNase 1 in the presence and absence of ethidium bromide was tested. Figure 4 shows that the time course of the liberation of acid-soluble products from the DNA in the presence of spermine is a sigmoidal curve whereas in absence of spermine a rectangular hyperbolic curve is obtained. These results may reflect that the DNA is collapsed into a condensed state by spermine. The compact structure of spermine-DNA complex may be protected from the hydrolytic attack of DNase 1 at the short period of incubation. Once the compact DNA structure is relaxed, the susceptibility of DNA to DNase 1 may be increased fast cooperatively. In case of the absence of spermine, the DNA is more fully extended compared to the DNA in presence of spermine and may be more susceptible to the hydrolysis by the DNase 1.

The present study, showing difference between 'in presence' and 'in absence' of spermine in ethidium-dependent susceptibility of DNA for DNase 1 correlated with the binding patterns of ethidium bromide to DNA, will be useful for the probing and understanding of the profile of structural transition of the DNA induced by spermine.

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